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Chemically Defined Medium for *Legionella pneumophila* Growth

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A chemically defined medium containing 18 amino acids, inorganic salts, rhamnose, choline, and ferric pyrophosphate has been developed. The final concentrations of salts and amino acids were modeled after yeast extract. This medium supported the growth of four serogroups of *Legionella pneumophila*. Growth in shake cultures at 37°C produced a lag time of approximately 5 h and a generation time of 4 h with a maximum growth yield of 10^9 colony-forming units per ml. A soluble brown pigment was observed in the stationary phase of growth. The optimal pH was 6.3. Rhamnose and choline were stimulatory; arginine, serine, threonine, cysteine, valine, and methionine were essential. Supplemental iron was not required to attain maximum growth, but iron deprivation caused an extended lag phase.

Most work with *Legionella pneumophila* has been done on various solid media (1, 2). Although these media are supportive of good growth, they are not satisfactory for use in controlled metabolic experiments. A chemically defined liquid medium is preferred for physiological studies, since optimal control of growth parameters can be established. Several investigators have described chemically defined media (4, 5); however, we felt that these media could be improved upon since they either were complicated to prepare or required a large initial inoculum that produced only one or two generations. We have previously reported a complex medium that was based on filtered yeast extract (5). We therefore chose to develop a chemically defined medium modeled after the yeast extract of BBL Microbiology Systems (6). In the present communication we describe this medium and also demonstrate that carbohydrates and other supplemental carbon sources and iron play a greater role than previously thought.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* strains Philadelphia (no. 3), Togus (no. 2), Bloomington (no. 2), and Los Angeles (no. 4) were obtained from the Center for Disease Control, Atlanta, Ga., on charcoal-yeast extract agar slants.

Plating medium. Charcoal-yeast extract agar, developed by Feeley et al. (1), was used for routine culturing and plate counts.

Stock cultures. Cultures were maintained in hen yolk sac membrane homogenates stored at −70°C. These cultures were one passage from original slants received from the Center for Disease Control.

Dilution blanks. All dilutions were made in 0.85% saline (pH 7.0).

Growth conditions. Inocula were prepared from cultures grown for 72 h on charcoal-yeast extract agar plates. Cells were harvested from plates with normal saline, washed once, and suspended in 10 ml of the same diluent.

Acid-washed 1-liter Erlenmeyer flasks containing 400 ml of medium were inoculated with sufficient cells to obtain an initial cell count of 7×10^6 colony-forming units per ml. Cultures were incubated in a controlled-environment incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C at 100 *oscillations per min without* CO_2.

All plates were incubated at 37°C in a moist atmosphere without CO_2.

Preparation of the chemically defined medium. The composition of the medium is listed in Table 1. The components were added to deionized, distilled water; choline, rhamnose, and ferric pyrophosphate, when used, were added after the pH had been adjusted to 6.3 with 1 N NaOH. The final pH of the medium was 6.3 ± 0.02. The medium was then sterilized by filtration through a 0.45-μm filter.

Soluble ferric pyrophosphate was obtained from the Center for Disease Control. Amino acids and other organic compounds were from Sigma Chemical Co. (St Louis, Mo.). All other chemicals were of analytical grade and obtained from several commercial sources.

Other procedures. Growth and pigment production were measured turbidimetrically on a model DB-G Beckman spectrophotometer at 660 and 392 nm, respectively. Colony-forming units were counted after 72 h of incubation. The pH of all media and samples was measured with a Beckman Zeromatic pH meter standardized with Beckman pH standard buffer (pH 7.0; 25°C). Microscopic observations were made with a Zeiss epi-illuminated fluorescence microscope.

RESULTS

Growth parameters in chemically defined liquid medium. Figure 1 shows the growth characteristics of *L. pneumophila* Philadelphia 3. Plate counts from samples removed

TABLE 1. *Composition of the chemically defined medium*

Ingredient	mg/liter
L-Arginine	350
L-Aspartic acid	510
L-Cystine[a]	160
L-Cysteine hydrochloride	400
Glycine	180
L-Glutamic acid	600
L-Histidine	150
L-Isoleucine	470
L-Leucine	640
L-Lysine	650
L-Methionine	200
L-Phenylalanine	350
L-Proline	115
L-Serine	650
L-Threonine	330
L-Tryptophan	100
L-Tyrosine[b]	400
L-Valine	480
Choline[c]	20
NH_4Cl	315
NaCl	50
$CaCl_2$	20
KH_2PO_4	1,180
$MgSO_4$	70
Ferric pyrophosphate (soluble)[c]	250
Rhamnose[c]	1,600

[a] L-Cystine was first dissolved in a small volume of 1 N HCl.

[b] L-Tyrosine was first dissolved in a small volume of 1 N NaOH.

[c] *Choline, rhamnose, and ferric pyrophosphate were* added after the pH was adjusted to 6.3. The final pH was 6.3 ± 0.02.

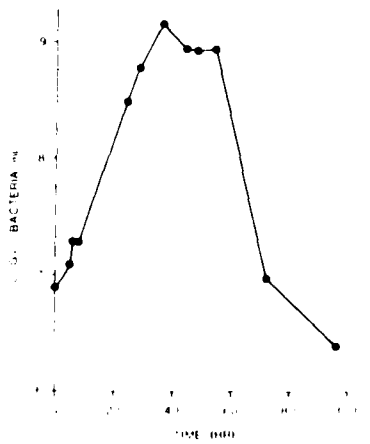

FIG. 1. *Growth of L. pneumophila in chemically defined medium.*

FIG. 2. *Appearance of L. pneumophila in chemically defined medium at 40 h (×1,000).*

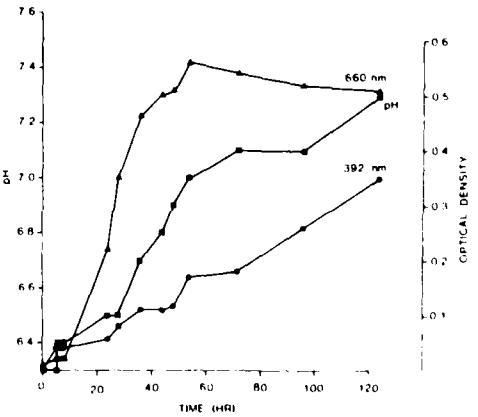

FIG. 3. *Kinetics of growth, pH, and pigment of L. pneumophila in chemically defined medium.*

at 5, 6, 7, and 8 h postinoculation indicate that the strain has a lag phase of less than 5 h. The logarithmic growth proceeded for 31 h and attained a maximum cell population at 37 h of 1.4×10^9 colony-forming units per ml with an average generation time of 4 h. The stationary phase continued for an additional 18 h. At 96 h, the population was 2.4×10^6 colony-forming units per ml. Microscopic observations at 40 h (Fig. 2) revealed predominantly diplobacillary forms with tapered ends. Vacuolated long chains were occasionally observed, but only appeared at a rate of one to two per oil immersion field. Figure 3 illustrates the correlation of bacterial growth to pH and pigment production. Pigment

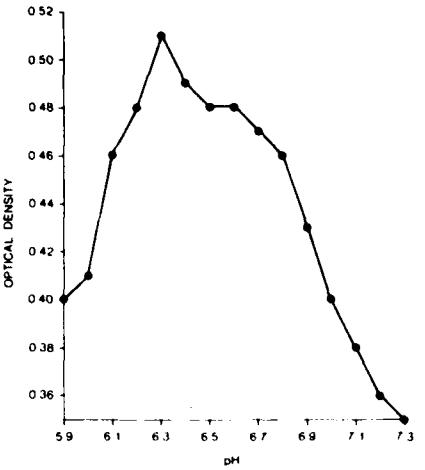

FIG. 4. *Effect of initial pH on the maximum optical density at 38 h.*

production (at 392 nm) occurred as soon as logarithmic growth began and gradually increased throughout the growth cycle. The only deviation from this was seen during the stationary phase at 48 h. The pH increased over the entire growth cycle; however, the most rapid increase was seen between 24 and 56 h. Optical density measurement at 660 nm indicated that during the stationary and death phases there was very little cell lysis.

Determination of optimal pH. Figure 4 shows the effect of initial pH on the maximum growth obtained at 38 h. Optimal growth was attained at an initial pH of 6.3; however, pigment production was delayed at this value, compared to a pH of 6.9.

Growth requirements for L-valine, L-methionine, L-arginine, L-serine, L-threonine and L-cysteine. *L. pneumophila* has definitive requirements for the amino acids L-cysteine, L-serine, L-methionine, L-valine, L-arginine, and L-threonine (Table 2). It was found that when L-cysteine was deleted from the medium there was only moderate growth as compared to the control. The removal of cystine from the medium had no effect on growth; however, when both cysteine and cystine were deleted from the medium the growth obtained was only slight. This small percentage of growth may be due to utilization of endogenous amino acids in the cell. Threonine and serine have been proposed as energy sources for *L. pneumophila* (3). Arginine and valine in our medium were found to be essential for the initiation of growth. It appears that these two amino acids, like threonine and serine, also are probable energy sources for *Legionella*.

TABLE 2. *Effect of deletion of certain amino acids on the growth of L. pneumophila in a chemically defined medium*

Deleted amino acid	% of control optical density (660 nm) at 40 h
Arginine	12 ± 0.8
Cysteine	34 ± 5.4
Cystine	100 ± 0
Cystine + cysteine	1.6 ± 0.3
Methionine	10 ± 0.5
Serine	9 ± 0.3
Threonine	3 ± 0.5
Valine	4 ± 1.2

TABLE 3. *Effect of supplemental ferric pyrophosphate on the growth of L. pneumophila in a chemically defined medium*

Iron (mg/liter)	Optical density (660 nm) at:	
	24 h	48 h
0	0.17	0.54
50	0.26	0.52
100	0.28	0.54
250	0.31	0.51
350	0.30	0.53
500	0.28	0.53
750	0.25	0.51
1,000	0.21	0.50

Effect of supplemental ferric pyrophosphate. Iron concentration appears to be a factor in determining the rate of growth as seen by 24-h readings (Table 3). Comparison of the readings taken after 24 and 48 h of incubation indicates that the generation time was altered as the iron concentration changed. This was particularly evident in the sample without supplemental iron, indicating that iron is not an essential growth factor, but is necessary for initial rapid growth. This effect on growth is due to a shortened lag time, which is dependent on the concentration of ferric pyrophosphate.

Effect of choline and carbohydrates. Table 4 indicates that choline stimulates the growth of *L. pneumophila*. Rhamnose was the only carbohydrate examined which appeared to have a synergistic effect with choline. It was also noted that the choice of carbohydrate affected the alkalinity of the medium as seen by the 40-h recordings (Table 4).

Effect of α-ketoglutaric acid and pyruvic acid. Both α-ketoglutaric and pyruvic acids have been used in an attempt to stimulate the growth of *L. pneumophila* (4, 7). Both acids were inhibitory to growth of *Legionella* in this synthetic medium (Table 5).

TABLE 4. *Effect of choline and carbohydrates on the growth of L. pneumophila in a chemically defined medium*[a]

Choline (20 mg/liter)	Added compound (1.6 g/liter)	pH at 40 h	% of control optical density (660 nm) at 40 h
−	None	7.20	100
+	None	7.20	125 ± 1.08
+	Dextrose	6.80	112 ± 1.04
+	Galactose	7.20	116 ± 1.25
+	Maltose	6.75	112 ± 1.04
+	Rhamnose	7.35	131 ± 1.11
+	Sucrose	7.10	113 ± 0.63
+	Xylose	7.25	120 ± 0.65

[a] Initial pH of all samples was 6.3 ± 0.02.

TABLE 5. *Effect of α-ketoglutaric acid and pyruvic acid on the growth of L. pneumophila in a chemically defined medium*[a]

Compound	Concn (%)	% of control optical density (660 nm) at 40 h
α-Ketoglutaric acid	0.1	89.0 ± 2.00
	0.5	89.6 ± 2.40
Pyruvic acid	0.1	2.1 ± 0.05
	0.5	1.0 ± 0.03

[a] Initial pH of all samples was 6.3 ± 0.02.

DISCUSSION

Preliminary characterizations of *L. pneumophila* suggested rather fastidious nutrient requirements (1, 2, 4). These requirements, however, may not be as stringent as initially indicated (3, 7). Warren and Miller (7) successfully grew *L. pneumophila* in what can be considered a simple medium. We have obtained similar results with a less complicated medium. Growth was obtained with 18 amino acids, 6 of which proved to be essential. The amino acids phenylalanine, tyrosine, aspartic acid, and cystine could be deleted without an appreciable effect on growth (unpublished data). Cystine could only partially replace the cysteine requirement even in the presence of high concentrations of methionine. Other nutritional requirements seemed to be minimal. Vitamins, purines, pyrimidine, and coenzymes were not employed because we had found no effect on the rate of growth at various concentrations. This would indicate, as previously suggested (5), that *L. pneumophila* has the capability to synthesize all its required vitamins and vitamin-like compounds.

Previous reports indicated that *L. pneumophila* has a requirement for ferric ion or hemoglobin (1, 2, 4). However, Warren and Miller (7) demonstrated that supplemental ferric pyrophosphate is not necessary for growth. More importantly, we showed that *L. pneumophila* can grow over a broad range of iron concentrations, providing further evidence of the metabolic diversity of this organism. Like Warren and Miller (7), we observed an increased lag time under conditions of iron deprivation. This may reflect the necessity of the organism to generate iron transport compounds for trace iron utilization. Warren and Miller (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D67, p. 49) indicated that under low iron concentrations iron siderophores were produced. Additionally, we observed that with low iron concentrations, cellular morphology was affected. Under these conditions, we observed a short coccobacillary form, whereas high iron concentrations produced long slender rods, but no filaments.

Rhamnose appeared to enhance the growth of *L. pneumophila*; however, the reasons for this effect have not been established. Rhamnose and other carbohydrates may serve as carbon and energy sources for growth. By contributing to the metabolism in this manner, carbohydrates may stimulate the rapid deamination of certain amino acids in the medium. This enhanced deamination could explain both the increase in pH and the stimulation of growth in the presence of rhamnose. This stimulation may indicate that a simple nitrogen source is required for metabolism.

In an effort to find other compounds that would stimulate growth we incorporated α-ketoglutaric and pyruvic acids into the medium. The inhibitory effect that we observed was not expected, since others have not had similar results (4, 7). The concentrations of other constituents in our synthetic medium are different from those previously reported. This variation in concentration may provide a more sensitive system for feedback inhibition of α-ketoglutaric and pyruvic acids.

These observations are helpful in elucidating the physiology of an organism that has simple nutritional requirements for growth, but a complex metabolism. This is not unexpected, considering the ubiquitous nature of *L. pneumophila*. The capability to survive in an austere environment demands that an organism be adaptable and have simple nutritional requirements.

Unfortunately, this metabolic adaptability makes the isolation and physiological studies of the organism difficult. Organisms isolated from clinical sources will more than likely be shown to have different nutritional requirements from those isolated from the environment. Hopefully, by further characterization of this medium the basic metabolic requirements of *L. pneumophila*

can be understood, and this may contribute to improved clinical and environmental isolation techniques.

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